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31EG4 mammary epithelial cells (mec) were rown as monolayers on filters in the presence of dexamethasone to induce polarization and formation of high transepithelial resistance. RT-PCR showed the presence of CFTR and ENaC message. Immunomicroscopy showed apical staining for both ENaC and CFTR. Intracellular recordings demonstrated the functional presence of ENaC and CFTR on the MEC apical membrane. Apical amiloride hyperpolarized the apical membrane potential (V_A), increased the ratio of apical to basolateral membrane resistance (R_A/R_B) and decreased short circuit current (I_{SC}) and transepithelial conductance (G_T). Forskolin depolarized V_A, decreased R_A/R_B by and increased I_{SC} and G_T. Whole cell patch clamp measurements demonstrated the presence of amiloride-inhibitable Na currents and forskolin-stimulated Cl currents. 31EG4 monolayers secreted or absorbed fluid (1-2 $\mu l/cm^2 \cdot hr$) in the basal state, and forskolin increased fluid flow in the direction it was moving in the basal state. All forskolin-stimulated responses (V_A, G_T, I_{SC}, fluid) were blocked by NPPB. We conclude that MEC transport Na and Cl through apical ENaC and CFTR, respectively. 31EG4 cells should prove useful for testing the regulation of ENaC by CFTR- important in cystic fibrosis. ENaC and CFTR are likely important in controlling the ion and water composition of milk.

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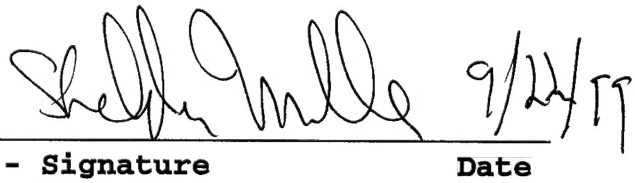

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INTRODUCTION

The goal of the present work was to develop a model system to study the mechanisms of fluid transport across mammary epithelia. In addition we also examined whether the epithelial ion Cl and Na channels (cystic fibrosis transmembrane conductance regulator, CFTR, and ENaC, respectively) contribute to the ion and fluid transport properties of the mammary epithelial cell line 31EG4. We reasoned that since the mammary gland is a modified sweat gland (Pfeifer et al., 1999. Van Der Putte et al., 1995), embryologically derived from sweat glands, with similar functions it may utilize many of the same plasma membrane transport proteins to carry out those functions. Since sweat duct cells express high levels of both CFTR and ENaC to allow reabsorption of salt from the fluid that flows down the duct (Soos, 1995), 31EG4 cells might also express these ion channels and use them to control the ionic and fluid composition of milk. We used PCR to identify the genes, western blot and immunomicroscopy to identify the proteins and transepithelial, microelectrode and patch clamp electrophysiology to demonstrate the appropriate ionic currents. Fluid transport was measured using a capacitance probe method to determine whether ENaC and CFTR also play roles in the transport of fluid across the monolayers.

BODY

The mammary gland is a branched, convoluted organ with acinar cells that secrete macromolecules (milk proteins, lactose, fats), salts (including Na, K, Ca, Cl and phosphate) and water and duct cells that likely modify the milk as it passes out to the nipple. Human milk contains roughly 200 mM lactose, 7 mM Na, 13 mM K, 8 mM Ca, and 12 mM Cl and is mildly acidic (pH 6.8) (Cuthbertson., 1999). It has been proposed that the initial secretion of milk by the acinar cells occurs as an isotonic fluid (2), largely driven by the production and then secretion of lactose and the osmotically driven water. The duct cells likely modify this fluid as it moves along the duct. The specific mechanisms involved in generating the macromolecular secretions and ionic and water transport have not been well described, but it has been proposed that fluid is secreted largely driven by lactose secretion into Golgi-derived vesicles that fuse with the apical plasma membrane to release the fluid. It is possible that the relatively low ionic content of milk is generated and/or maintained by the reabsorptive properties of the acinar and/or duct cells, but the specific ion channels and other transporters involved have not been described. Experiments on primary cultures of mouse mammary epithelial cells has shown that they actively absorbed Na in a process that was inhibited by apical addition of μM concentrations of amiloride (Bisbee, 1979), the well known blocker of the epithelial Na channel (ENaC; see Mirshahi, M., etal., 1999.) and stimulated by the lactogenic hormone prolactin (Bisbee, 1979). There was no statistically significant active transport of Cl, though in the short-circuited state these cultures appeared to actively absorb (unidentified) anions or secrete other cations because the rate of Na absorption was larger than the measured short circuit current.

Technical objectives 1-2

PCR, westerns and immunocytochemistry

PCR and western blot analysis were performed on 31EG4 cells grown to confluence in the presence of dexamethasone to increase differentiation of the cells. Results from PCR experiments are summarized in Fig. 1, where it is shown that message for both ENaC and CFTR was present in 31EG4 cells. Western blots (Fig. 2) for CFTR showed a typical band at approximately 170 kDa, and for ENaC α-subunit at approximately 85 kDa, indicating that both these proteins were also being made.

Immunomicroscopy was performed on 31EG4 cells grown to confluence on filters using the same anti-CFTR antibody and an antibody to the α-subunit of ENaC. Typical results from these experiments are shown in Fig. 3.

Technical objectives 3 and 5

Transepithelial and intracellular electrophysiology

As part of a project to determine how mammary cysts accumulate fluid, we began a study of the ion transport properties of the mammary epithelial cell line 31EG4. This cell line forms well polarized monolayers with tight junctions that are regulated in their "tightness" by the glucocorticoid dexamethasone (Zettl etal., 1992): dexamethasone causes the transepithelial resistance (R_T) to increase from 100 - 300 $\Omega \cdot cm^2$ to > 700 - 1000 $\Omega \cdot cm^2$ (Table 1), and the tight junction proteins ZO-1 and filamentous actin appear to become more "organized". It is thought that this regulation of the tight junctions recapitulates the transformation of mammary gland cells from the leaky epithelium characteristic of the resting condition to the tight epithelium characteristic of the lactating gland that must maintain the large transepithelial (milk to blood) concentration gradients for ions and macromolecules. Coincident with the increase in R_T, 31EG4 cells also express Na/H exchange and Na/HCO_3 cotransport activity in the basolateral, but not the apical, membrane (Sjaastad etal.,1993).

31EG4 monolayers were mounted in Ussing chambers and then treated with amiloride (to block ENaC) followed by forskolin (to stimulate cAMP production and activation of PKA) and DPC (to block CFTR). As shown in Fig. 4, 10 μM amiloride in the apical solution caused I_{SC} to decrease. In the presence of apical amiloride, forskolin (10 μM) increased I_{SC}, and subsequent addition of apical DPC (500 μM) decreased I_{SC}. These treatments were repeated several times with similar results. In four experiments on different cultures, amiloride decreased I_{SC} by an average of 2.5 $\pm$ 0.7 $\mu A/cm^2$; in the presence of amiloride, forskolin increased I_{SC} by 7.8 $\pm$ 1.4 $\mu A/cm^2$ and subsequent addition of DPC decrease I_{SC} by 5.5 $\pm$ 0.8 $\mu A/cm^2$.

Similar experiments were performed using a combination of intracellular microelectrode and transepithelial electrophysiological measurements. As shown in Fig. 5 and summarized in Table 1, these resting cells had a mean R_T of 857 $\Omega \cdot cm^2$ and mean TEP = - 4.8 mV. The apical side is negative relative to the basal side since V_B is more hyperpolarized than V_A by 4.8

mV. The ratio of the apical to basolateral membrane resistances was always larger than 1, indicating that the apical membrane resistance (R_A) was larger than that of the basolateral membrane (R_B). A cocktail of forskolin, CPT-cAMP and IBMX caused a rapid and reversible increase in TEP and drop in R_T (Fig. 5); these changes of transepithelial parameters were accompanied by a depolarization of both V_A and V_B by >10 mV and decrease of the R_A/R_B ratio by about a factor of two (Fig 5 and Table 2). Using Ohm's law it was calculated from the summary data in Table 1 that the equivalent short circuit current of the resting monolayer was 5.6 $\mu A/cm^2$, and this increased to 10.2 $\mu A/cm^2$ following treatment with the cAMP-raising cocktail, similar in magnitude to the forskolin-stimulated increase in I_{SC} presented above (Fig. 3). These results are consistent with the hypothesis that increasing intracellular cAMP activates apical CFTR and increases Cl or other anion transport from the basolateral to the apical surface at a rate of about 5 $\mu A/cm^2$.

Since the CFTR-blocker DPC appeared to block the forskolin-induced increases in I_{SC} (Fig. 3), it was expected that the cAMP-induced changes in membrane voltage and resistance would also be blocked by DPC or NPPB, another CFTR-blocker. As shown in Fig. 6, forskolin had little effect on R_T, R_A/R_B, TEP, V_A and V_B in the presence of apical NPPB. Thus, forskolin depolarized V_A and V_B, decreased R_A/R_B and R_T and increased TEP. Apical NPPB blocked these effects, consistent with the presence of apical CFTR. A summary of other, similar experiments is shown in Table 2. We have also used these data to perform equivalent circuit analysis which is presented below.

Whole cell patch clamp measurements were also performed on 31EG4 cells. As shown in Fig. 7A, forskolin caused a shift in reversal potential from approximately -5 mV to -20 mV and increased cellular conductance. Further treatment with DPC caused the reversal potential and the conductance to return to the resting, baseline level. A summary of the effects of forskolin and DPC on 31EG4 cell conductance is shown in Fig. 7B.

Similar experiments were performed to test for amiloride-sensitive changes in cellular voltages and currents. Results from a typical microelectrode experiment are shown in Fig. 8. Apically added amiloride caused TEP to decrease and R_T to increase. There was also a large increase in R_A/R_B accompanied by a large hyperpolarization of V_A and V_B. These changes in electrophysiology were rapidly reversed when amiloride was washed from the apical solution. Using Ohm's law and the summary data in Table 1 it was calculated from that the equivalent short circuit current of the resting monolayer was 5.6 $\mu A/cm^2$, which decreased to 1.8 $\mu A/cm^2$ following treatment with the cAMP-raising cocktail, similar in magnitude to the amiloride-induced decrease in I_{SC} presented above (Fig. 3). Addition of 20 μM amiloride to the basal solution caused small (<5 mV) depolarizations to V_A and V_B with no change in TEP, a 30% drop in R_A/R_B and a 5% increase in R_T (not shown). The effects of amiloride on the basolateral Na/H exchanger may have secondarily caused these effects and resulting changes in cell pH (Sjaastad et al,1993). The effects of apically added amiloride are consistent with the blockade of Na entry through apical ENaC. Average results from similar experiments have been summarized in Table 2. We have performed equivalent circuit analysis using these data immediately below.

Whole cell patch clamp experiments were also used in an attempt to identify amiloride-sensitive currents. Currents were quite small in the resting state (see Fig. 9A), so inhibitory

effects of amiloride were difficult to identify in every cell. Results from four experiments are shown in Fig. 9. Results from one of these experiments in which cells were incubated in a NaCl-containing Ringer's solution (see Methods) are shown in Fig. 9A and the two experiments are summarized in Fig. 9B: the I-V curve showed small, linear currents (whole cell conductance =16.4 nS, reversal potential = 10 mV). Both current and conductance were inhibited by amiloride, and the reversal potential shifted in the positive direction to - 5 mV. Similar experiments were also performed on cells in which the bathing solution was Na gluconate Ringer's. In these two experiments (summarized in Fig. 9B), amiloride had much smaller effects on currents and conductances of the cells.

Calculating resistances and EMF's for apical and basolateral membranes and shunt

The microelectrode data presented above is summarized in Table 1 calculate resistances and EMF's for the apical and basolateral membranes and for the shunt for 31EG4 cells in control and during amiloride and cAMP cocktail conditions. These calculations have been summarized in Table 2.

Comparison of amiloride effects under control and cAMP-treated conditions

Previous experiments have shown increased Na absorption in cystic fibrosis tissues (Stutts et al., 1995.), and heterologous expression of CFTR is known to decrease the activity of ENaC in both frog oocytes and MDCK cells (Chabot, H et al., 1999.). Since 31EG4 cells appeared to express both CFTR and ENaC in the native cells, we tested whether cAMP treatment would alter the activity of ENaC. Typical experiments are shown in Fig. 11. In the first part of the experiment, amiloride caused a typical large hyperpolarization of V_A (and V_B), large increase in R_A/R_B and increase in R_T. TEP decreased since the hyperpolarization was larger at the apical compared to the basolateral membranes. Subsequent addition of the cAMP cocktail caused a very large depolarization of V_A (and V_B), drop in R_A/R_B (changes that were larger than those exhibited in the absence of amiloride) (Fig. 11). Addition of cAMP cocktail to the amiloride-treated cells also caused TEP to increase and R_T to decrease. These results showed that the cells were responsive to cAMP in the presence of amiloride.

In contrast, apical amiloride caused much smaller electrophysiological changes in the presence of cAMP stimulation. As shown in Fig. 12, V_A and V_B hyperpolarized by only 5 mV, and R_A/R_B, R_T and TEP hardly changed in the presence of cAMP. Thus, there was little detectable effect of amiloride in the cAMP-treated cells. A summary of the effects of amiloride in control and cAMP-treated monolayers is shown in Table 3.

Technical objective 4

Fluid transport

31EG4 monolayers grown on filters were mounted in a specially designed fluid transport chamber (Hughes et al, 1984; Jiang et al, 1993), and the rates of transepithelial fluid movement along with TEP and R_T were measured in the baseline, control condition and then following the addition of the cAMP-stimulating cocktail. The direction of fluid transport was variable. As shown in Figs 12A and B and summarized in Fig. 13, in three monolayers, baseline fluid

transport was in the secretory direction at rates of 0.5 - 2.0 $\mu l/cm^2 \cdot hr$, while in two others the monolayers absorbed fluid at roughly the same rates. Addition of the cAMP cocktail caused, in addition to the typical drop in R_T and increase in TEP, an increase in fluid secretion from 2 to 8 $\mu l/cm^2 \cdot hr$ in two of the three monolayers that began secreting in the basal state. In three other experiments cAMP cocktail stimulated fluid absorption from 6 to 11 $\mu l/cm^2 \cdot hr$ (Fig. 12B and 13).

DISCUSSION

CFTR and ENaC and ion permeation in 31EG4 mouse mammary epithelial cells

A major finding of this paper is that the normal (ie., non-transformed) mammary epithelial cell line 31EG4 expresses CFTR and ENaC in the apical membranes. Both CFTR and ENaC message were identified, and western blots showed that proteins with the expected molecular sizes were present in these cells. Immunomicroscopy indicated that both channels were either in the apical membrane or located very close to the apical membrane.

Although the immunomicroscopy was inconclusive regarding the precise membrane locations of CFTR and ENaC, electrophysiology indicated that the channels were expressed in the apical membranes. In the short circuited condition or using open circuit TEP and R_T data to calculate equivalent short circuit current, 31EG4 cells exhibited currents of 2 to 6 $\mu A/cm^2$ that were blocked by apical application of 10 μM amiloride. Whole cell patch clamp measurements on cells incubated in NaCl Ringer's solution also demonstrated amiloride-inhibited currents and conductance. Microelectrode experiments showed that amiloride caused a very large increase in R_A/R_B consistent with blocking apical ENaC. The observation that amiloride increased $R_A/R_B = 30$ (Table 1) is also consistent with the apical membrane of resting cells being highly selective to Na. This will be discussed in more detail below.

Electrophysiological experiments showed that increasing intracellular cAMP also increased anion (likely Cl or HCO_3) movements through apical CFTR. Thus, cAMP increased basolateral-to-apical membrane anion current by approximately 6 $\mu A/cm^2$, accompanied by reductions in R_T and R_A/R_B; these changes were blocked by apical addition of DPC and NPPB, both of which are known to block CFTR (Walsh et al., 1999).

Calculating resistances and EMF's for apical and basolateral membranes and shunt

Our electrophysiological data were used to calculate the resistances and equivalent motive forces for each of the 31EG4 membranes in control and amiloride-treated conditions. In the control condition, R_A and R_B and E_A and E_B values were similar to those observed in many other epithelia. The relatively low value of R_S compared to R_A and R_B, and the very large effect of amiloride on R_A give rise to some interesting conclusions. (i) R_S is significantly smaller than both R_A and R_B, and total cellular resistance ($R_{cell} = R_A + R_B = 4118\ \Omega \cdot cm^2$) is more than four times larger than R_S (= 998 $\Omega \cdot cm^2$). Thus, the absolute resistance of R_S is high compared to many tissues with so called "leaky" low resistance tight junctions ($R_S \leq 100$ - 200 $\Omega \cdot cm^2$; Kovbasnjuk, O., et al., 1998), but the predominant pathway for current flow across 31EG 4

monolayers was through the shunt, similar to the condition of these leaky tissues. The importance of the shunt/tight junctions in transepithelial ion and fluid transport will be discussed in more detail below. (ii) The fact that the shunt was relatively leaky compared to the cellular pathway gave rise to the observations that amiloride increased R_A by almost 20-fold from 2528 to 48,400 $\Omega \cdot cm^2$, but only increased R_T by 14% from 857 to 979 $\Omega.cm^2$. (iii) In the amiloride-treated condition, almost all transepithelial current flow was through the shunt. (iv) The very large increase in R_A during amiloride treatment indicated that in the basal state there were few other active channels in the apical membrane. (v) The relative constancy of E_B and R_B values during amiloride treatment are consistent with the idea that amiloride almost exclusively acts to block apical ENaC.

Fluid transport and integrated cellular activity

Another major finding of these studies is that 31EG4 cells in the control condition transported fluid, and this was increased by cAMP (which opens CFTR) and blocked by NPPB (blocks CFTR). In 40 % of the monolayers, fluid transport in the basal state (no cAMP) was in the absorptive direction at an average rate of 2.2 $\mu l/cm^2 \cdot hr$. Since apical membranes of control cultures (no cAMP stimulation) had open ENaC but low CFTR activity, it might be proposed that fluid absorption in control cells was driven by the active transport of Na through apical ENaC and basolateral Na/K-ATPase, and accompanying anion transport was through the few open CFTR's and perhaps through the tight junctions driven by the apical-negative TEP.

In 60 % of the control monolayers, fluid transport was in the secretory direction (average = $\mu l/cm^2 \cdot hr$). As shown in Fig. 10A, this fluid secretion was stimulated by cAMP. One possible mechanism to explain this fluid secretion would involve active transport of Cl or HCO_3 through cAMP-activated CFTR, with accompanying Na following through the tight junctions driven by the TEP.

An interesting implication of these proposals is that net ion and fluid movements across intact monolayers depend critically on the properties of both the cellular and paracellular (tight junctions) pathways. In particular, net salt and fluid secretion will require Na movement through the tight junctions to accompany active Cl secretion through apical CFTR. In contrast, net salt and fluid absorption will require Na movement through ENaC and (based on the fact that NPPB blocked fluid absorption) Cl movement through CFTR. Because fluid secretion requires basal-to-apical Na transport through tight junctions, while fluid absorption requires that the tight junctions be relatively tight to Na to prevent Na leaking back across the epithelium, it seems likely that tight junctions may be regulated in some very interesting ways. Such regulation could explain why some monolayers absorbed fluid while others secreted fluid, and both secretion and absorption were stimulated by cAMP. Since the tight junction pathway of 31EG4 cells can be regulated by the presence or absence of the glucocorticoid dexamethasone (1μM),

and, in the presence of apical amiloride the shunt pathway is the dominant pathway for transepithelial current flow (Table 2), these cells offer the possibility of investigating the role of tight junctional integrity in ion and fluid transport.

Effects of cAMP on CFTR and ENaC

An interesting aspect of our experiments was that stimulation of cAMP production caused, in addition to the expected apparent increase in CFTR activity and consequent drop in apical membrane resistance, a decrease in the effects of amiloride on cellular potentials, TEP and R_T. This result may be explained in any of the three following ways: (i) cAMP may have changed the permeability properties of ENaC such that it still conducted Na, but it was no longer sensitive to amiloride. This would explain why some monolayers exhibited increased fluid absorption during cAMP treatment - ENaC was open, and CFTR was also, allowing Cl to follow Na and generate increased fluid flow - but there was little or no effect of amiloride on 31EG4 electrophysiology. (ii) Another possibility was that cAMP inhibited ENaC but (at least in some cases) opened another amiloride-independent Na channel in the apical membrane that would provide for Na absorption to drive the cAMP-stimulated fluid absorption that often occurred. (iii) In light of the work showing that CFTR may negatively regulate ENaC [e.g., in frog oocytes (Chabot., et al., 1999) and MDCK cells (Kunzelmann, et al.,1997), it also seems possible that our results could also be explained by activated CFTR inhibiting ENaC (and, following the logic of (ii), also opening an alternative Na channel in some monolayers). We noted in this regard that amiloride had much smaller effects on whole cell currents and conductances in cells bathed in Na gluconate Ringer's compared to results in NaCl Ringer's, consistent with active CFTR regulating ENaC. Further work will be required to determine whether any of these three scenarios is correct. 31EG4 cells may provide a good model cell type for studies of the interactions between CFTR and ENaC because both ion channels are expressed in their native state, and transfections of either or both channels is not required.

Mammary gland physiology

The present results on 31EG4 cells demonstrating amiloride-inhibitable Na transport are consistent with previous experiments showing the presence of amiloride-sensitive Na absorption across short circuited primary cultures of both midpregant and lactating mammary gland cells (Sjaastad et al., 1993). Since mammary gland cells are derived embryologically from sweat glands, and sweat ducts express high levels of both CFTR and ENaC in their apical membranes, our results may indicate that ENaC and CFTR of mammary duct cells are similarly involved in generating and/or maintaining the low Na and Cl concentrations of milk. Future experiments using immunomicroscopy and/or *in situ* hybridization may yield important insights about the roles of specific cell types that express CFTR and ENaC in intact mammary glands during pregnancy and lactation.

The present results indicate that 31EG4 cells, like sweat duct cells, absorb Na through ENaC. In addition, both 31EG4 cells and sweat duct cells transport Cl through CFTR. However, there are at least two differences between these two cell types. First, CFTR in apical membranes of sweat duct cells appears to be constitutively fully active, while CFTR in 31EG4 cells needed to be stimulated by cAMP to become active. Second, sweat ducts utilize CFTR to

absorb Cl, while 31EG4 cells appeared to use CFTR for both secretion and absorption. Thus electrophysiology showed changes in response to cAMP-elevating treatments consistent with Cl and/or HCO_3 secretion that were blocked by DPC; fluid secretion was similarly often stimulated by cAMP. Active anion secretion in the basal state may have been through those few CFTR's that were constitutively active, while cAMP stimulated secretion by activating CFTR.

The fact that sweat ducts use ENaC and CFTR only for ion absorption while 31EG4 cells seem to use ENaC for absorption but CFTR for either absorption or secretion is likely due to the electrochemical gradients for Cl and/or HCO_3 across the apical CFTR. The electrochemical gradients in turn will be determined by both the tightness of the tight junctions and the activities of other ion transporters. Of course, the activities of other Cl and/or HCO_3 transport pathways (e.g., anion exchange, NaK2Cl cotransport) will also be important for determining the electrochemical gradient for anions at the apical membrane and the direction of Cl and HCO_3 across apical CFTR.

These considerations emphasize the fact that while ENaC will always be involved in Na absorption across epithelial cells, CFTR may function to either absorb or secrete Cl and/or HCO_3, depending on the electrochemical gradients for these ions across the apical membranes of the epithelial cells. These gradients in turn will be determined in part by the activities of both ENaC and tight junctions, which will both affect V_A (and also TEP). Given the numerous potential interactions between among ion channels (and other transporters) and tight junctions, it is perhaps not surprising that intact 31EG4 monolayers exhibit quite variable responses to cAMP stimulation. Similar considerations are also relevant for ion secretion and absorption in other epithelia that express both CFTR and ENaC.

Key Research Accomplishments:

- Established a mammary epithelia cell line (31EG4) for fluid transport studies.
- Immunologically and physiologically localized CFTR and ENaC to the apical membrane of 31EG4.
- Measured fluid movement across 31EG4 and found that the rate of fluid transport can be altered by CFTR activity.

Reportable Outcomes:

- Manuscripts; Sasha Blaug, Kevin Hybiske , Connie Yu , Van Nguyen, Gary L. Firestone, Terry E. Machen and Sheldon S. Miller; **MAMMARY EPITHELIAL CELLS EXPRESS APICAL ENaC AND CFTR AND TRANSPORT FLUID.**

- Degrees obtained that are supported by this award; Sasha Blaug, PhD, May 2000.

- Funding applied for based on work supported by this award; Breast Cancer Research Program, State of California, University of California Office of the President, 1999.

- Employment or research opportunities applied for and supported for and/or received on experiences/training supported by this award.; Funds supported Sasha Blaug's dissertation work Van Nguyen's and Nedda Misherghi's undergraduate fluid transport work on 31EG4 mammary cells.

Conclusions:

Mammary epithelial cells (MEC) are e embryologically similar to sweat glands, so we tested whether MEC actively transported Na and Cl through ENaC and CFTR. 31EG4 cells (non -transformed, clonal mouse MEC) were grown as monolayers on filters in the presence of the synthetic glucocorticoid dexamethasone to induce polarization and formation of high transepithelial resistance (R_T = 700 - 2000 $\Omega \bullet cm^2$). RT-PCR showed the presence of CFTR and ENaC (α subunit) message. Immunomicroscopy showed apical staining for both ENaC (β subunit) and CFTR. Apical amiloride (10 μM) hyperpolarized the apical membrane potential (V_A, measured with microelectrodes; referenced to basolateral solution) by a mean of 13 mV to -54 mV, increased the ratio of apical to basolateral membrane resistance (R_A/R_B) by a factor of 3-11 and decreased short circuit current (I_{SC}) and transepithelial conductance (G_T) by 2.5 $\mu A/cm^2$ and 0.15 mS/cm^2, respectively. Forskolin depolarized V_A by 15 mV, decreased R_A/R_B by a factor of three and increased I_{SC} by 6 $\mu A/cm^2$ and G_T by 0.15 mS/cm^2. Whole cell patch clamp measurements demonstrated the presence of amiloride-inhibitable Na currents (linear I-V's; G_{Na} = 0.5 nS) and forskolin-stimulated Cl currents (linear I-V's; G_{Cl} = 24.7 nS). 31EG4 MEC monolayers also secreted or absorbed fluid (1-2 $\mu l/cm^2 \cdot hr$) in the basal state, and forskolin increased (by a factor of two) fluid flow (usually) in the secretory direction. We conclude that MEC transport Na and Cl through apical ENaC and CFTR, respectively, and cAMP stimulation of CFTR increases secretion of fluid. ENaC and CFTR are likely important in controlling the ion and water composition of milk. 31EG4 cells should prove useful for testing the regulation of ENaC by CFTR, an interaction that is important for understanding the pathway associated with cystic fibrosis.

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Untreated tissue (n=28)				
TEP (mV)	R_t (Ω•cm2)	V_A (mV)	V_B (mV)	R_A/R_B
-4.8 ±1.6	857 ±256	-41 ±11	-45 ±12	2.7 ±0.3

Amiloride (apical) (n=18 for 12 tissues)								
ΔTEP (mV)	ΔR_t (Ω•cm2)	ΔV_A (mV)	ΔV_B (mV)	R_A/R_B	R_A (Ω•cm2)	R_A * (Ω•cm2)	R_B (Ω•cm2)	R_S (Ω•cm2)
3.0±1.0	126±57	-16±6	-13±5	11±4.1	4158	16,940	1540	1051

cAMP (apical) (n=12 for 9 tissues)								
ΔTEP (mV	ΔR_t (Ω•cm2)	ΔV_A (mV)	ΔV_B (mV)	R_A/R_B	R_A (Ω•cm2)	R_A * (Ω•cm2)	R_B (Ω•cm2)	R_S (Ω•cm2)
-2.4±0.7	-156±45	15±4.6	13±4.0	0.82 ±0.67	2894	879	1072	1093

Table 1. Summary of effects of amiloride and cAMP on cellular (V_A, V_B and R_A/R_B) and transepithelial (TEP and R_T) electrophysiology. The top set of columns show baseline data recorded from cells in 28 different monolayers. The lower two sets of data summarize the effects of amiloride (17 cells, 10 monolayers) and cAMP cocktail (12 cells, 9 monolayers) on electrophysiology in terms of changes in V_A, V_B and R_A/R_B and TEP and R_T.

Untreated tissue (n=28)				
TEP (mV)	R_t (Ω•cm2)	V_A (mV)	V_B (mV)	R_A/R_B
-4.8 ±1.6	857 ±256	-41 ±11	-45 ±12	2.7 ±0.3

Amiloride (apical) (n=18 for 12 tissues)												
ΔTEP (mV)	ΔR_t (Ω•cm2)	ΔV_A (mV)	ΔV_B (mV)	R_A/R_B	R_A (Ω•cm2)	R_A * (Ω•cm2)	R_B (Ω•cm2)	R_S (Ω•cm2)	EA (mV)	EA* (mV)	EB (mV)	EB* (mV)
3.0±1.0	126±57	-16±6	-13±5	11±4.1	4158	16,940	1540	1051	-22	-27	-57	-60

cAMP (apical) (n=12 for 9 tissues)												
ΔTEP (mV)	ΔR_t (Ω•cm2)	ΔV_A (mV)	ΔV_B (mV)	R_A/R_B	R_A (Ω•cm2)	R_A * (Ω•cm2)	R_B (Ω•cm2)	R_S (Ω•cm2)	EA (mV)	EA* (mV)	EB (mV)	EB* (mV)
-2.4±0.7	-156±45	15±4.6	13±4.0	0.82 ±0.67	2894	879	1072	1093	-28	-20	-50	-39

Table 2. Summary of voltages, EMF's and R's for the membranes and junctions of 31EG4 mammary monolayers. Definitions given in Materials and Methods.

cAmp + Amiloride (apical) (n=4 for 4 tissues)				
ΔTEP (mV)	ΔR_t (Ω•cm2)	ΔV_A (mV)	ΔV_B (mV)	R_A/R_B
0.0 ±0.0	8.75 ± 7.4	-2.25 ± 1.47	-2.25 ± 1.47	0.25 ±0.43

Table 3. Summary of voltages, TEP and R's for the membranes and junctions of 31EG4 mammary monolayers. Tissue goes from cAMP on the apical bath to cAMP + Amiloride (20μM). Definitions given in Materials and Methods.

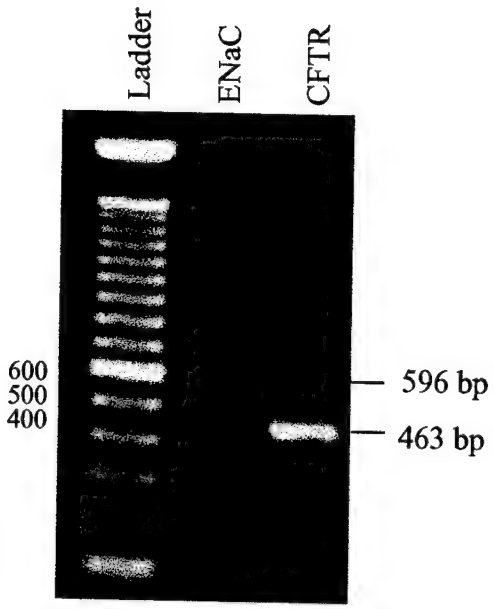

FIG. 1. PCR products using oligonucleotide primers designed to cover a 463 base pair region of CFTR and a 596 base pair region of ENaC. The amplified products were run on a 1.5% agarose gel and stained with ethidium bromide. The 100 base pair ladder is on the left.

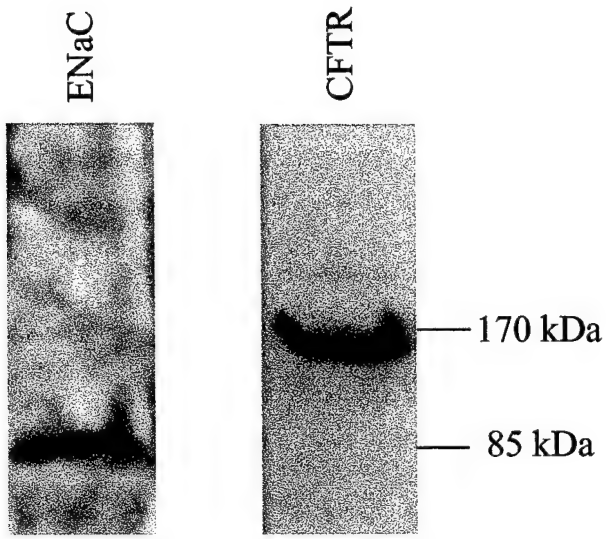

Fig. 2. Western blots for CFTR and ENaC. Western blots for CFTR and ENaC CFTR showed at approximately 170 kDa, and for ENaC β subunit at approximately 85 kDa, indicating that both these proteins were also being made.

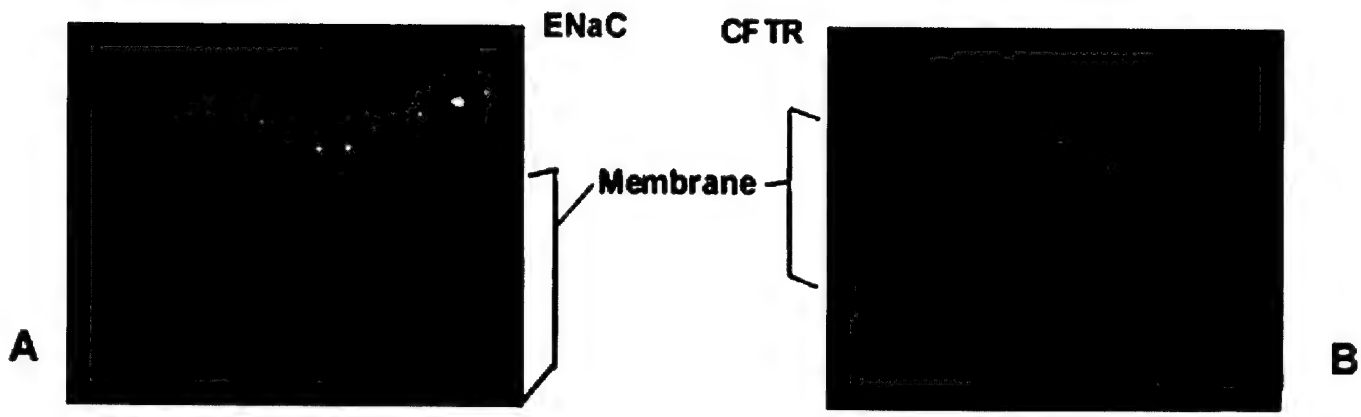

Fig. 3. Immunolocalization of EnaC (126x) (**A**) and CFTR(63x)(**B**) in 31EG4 mammary epithelial monolayers grown on filters.

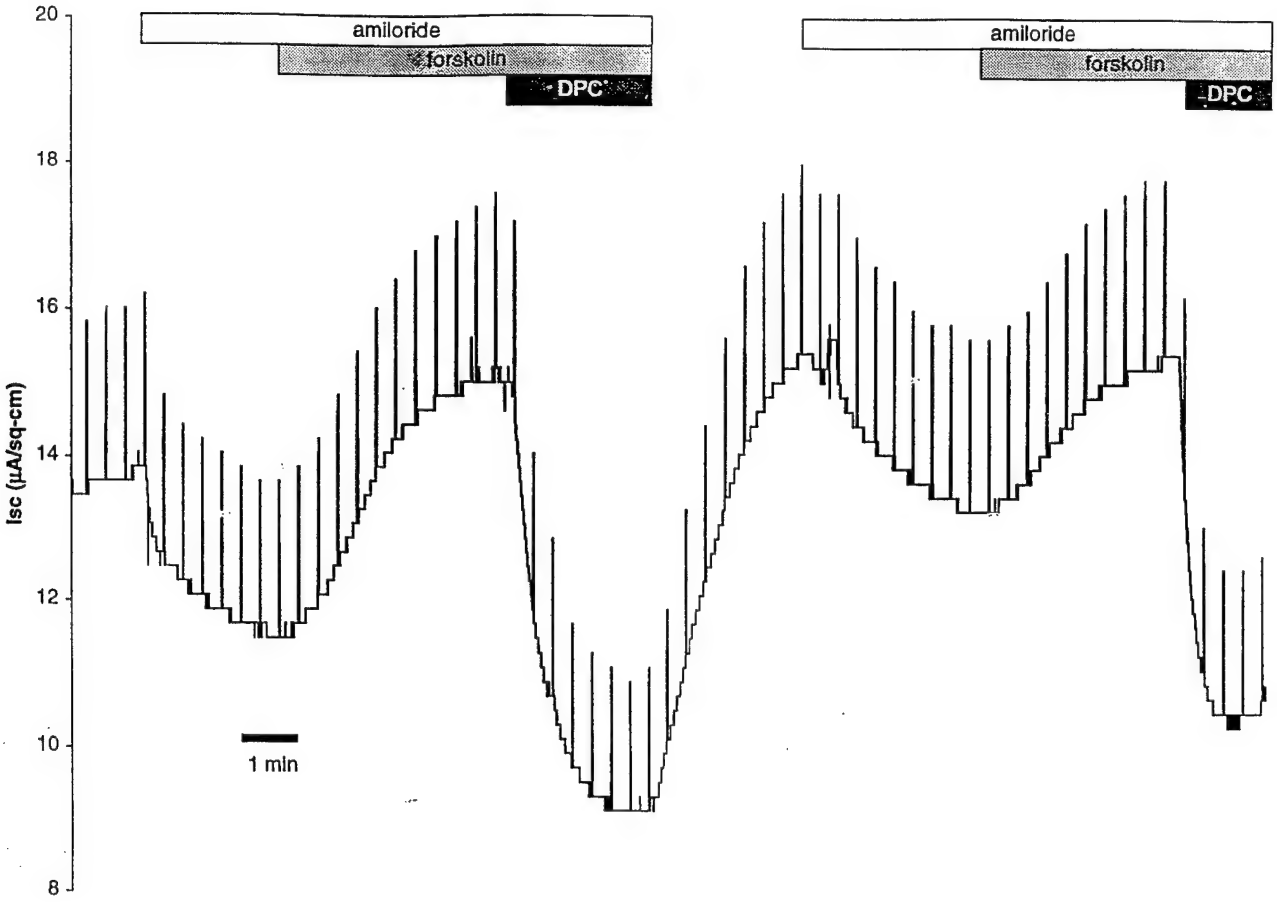

Fig. 4. Effects of amiloride, forskolin and DPC on I_{SC} of 31EG4 monolayers. Cells grown to confluency on filters were mounted in the chamber and I_{SC} was measured. Spikes in I_{SC} trace represent changes induced by briefly clamping epithelium to 5 mV. Apical addition of amiloride caused I_{SC} to decrease from about 13.5 $\mu A/cm^2$ to 11.5 $\mu A/cm^2$. Subsequent treatment with 10 μM forskolin (to increase cAMP) caused current to increase from about 13.5 $\mu A/cm^2$ to 15 $\mu A/cm^2$, and DPC reduced I_{SC} to about 9 $\mu A/cm2$. These effects were repeated in the second portion of the experiment, showing the reproducibility of the responses. Experiment is typical of 4 other experiments.

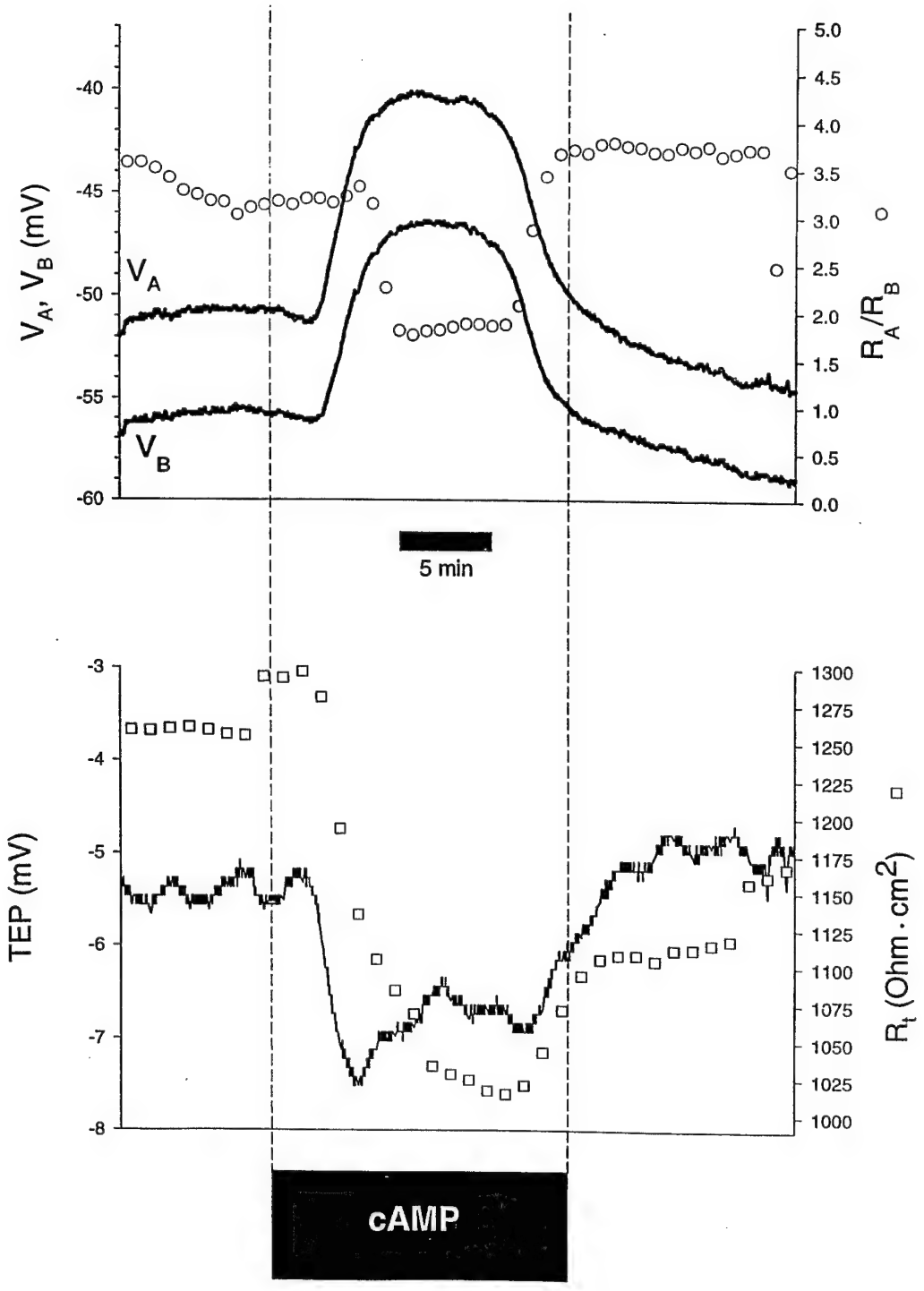

Fig. 5. Effects of cAMP cocktail on (**A**) cellular and (**B**) transepithelial electrophysiology in 31EG4 cells. All potentials measured with respect to the basolateral solution. The cAMP-raising cocktail (see Methods) caused, after a brief delay, depolarizations of both V_A and V_B by > 10 mV, while TEP increased; R_T and R_A/R_B both decreased. These results were consistent with cAMP opening Cl channels in the apical membrane.

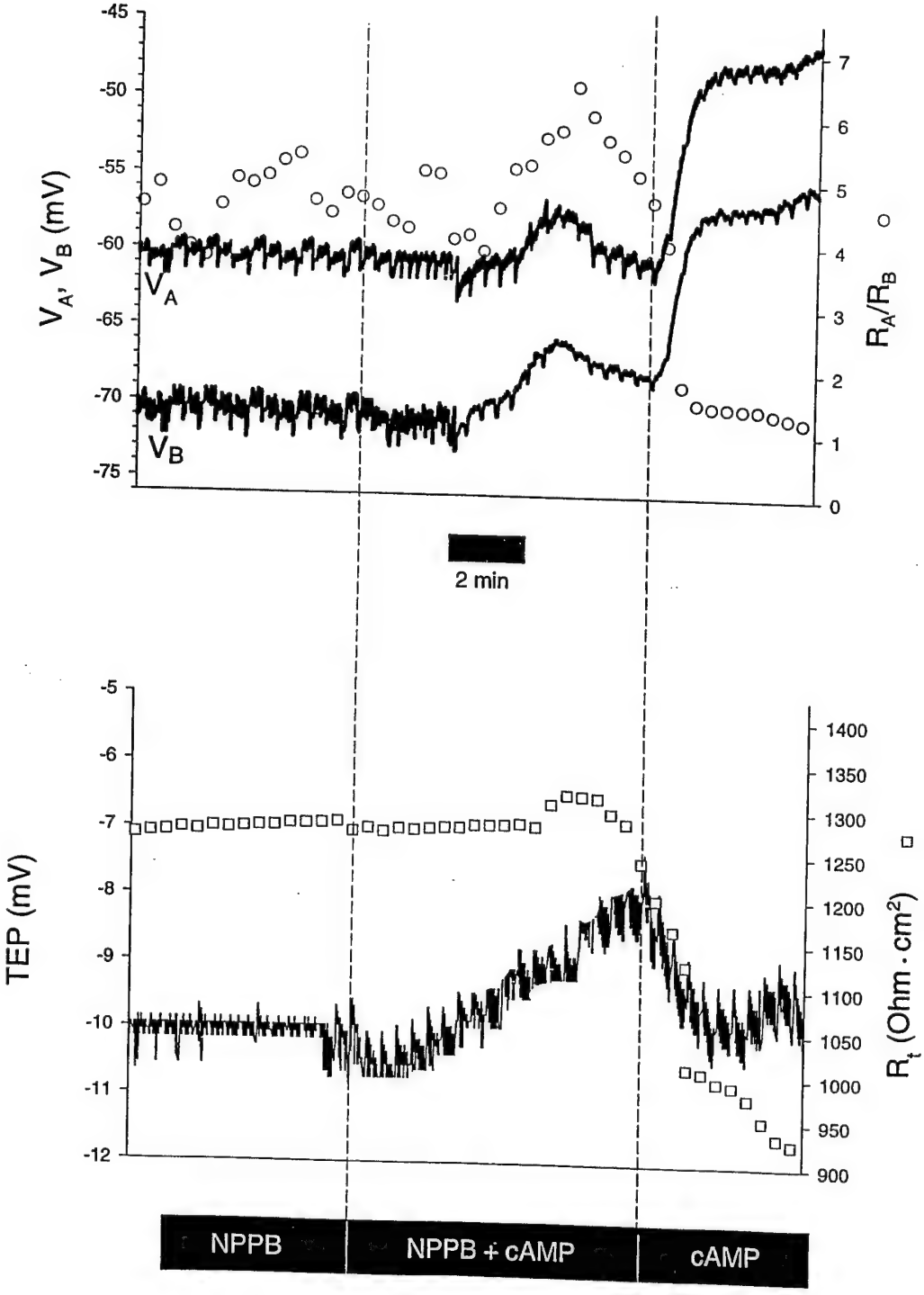

Fig. 6. NPPB blocks effects of cAMP on cellular and transepithelial electrophysiology in 31EG4 cells. In the presence of NPPB, the cAMP cocktail elicited only small effects on electrophysiology. Removal of NPPB caused V_A and V_B to depolarize by > 10 mV, while TEP increased and R_T and R_A/R_B both decreased.

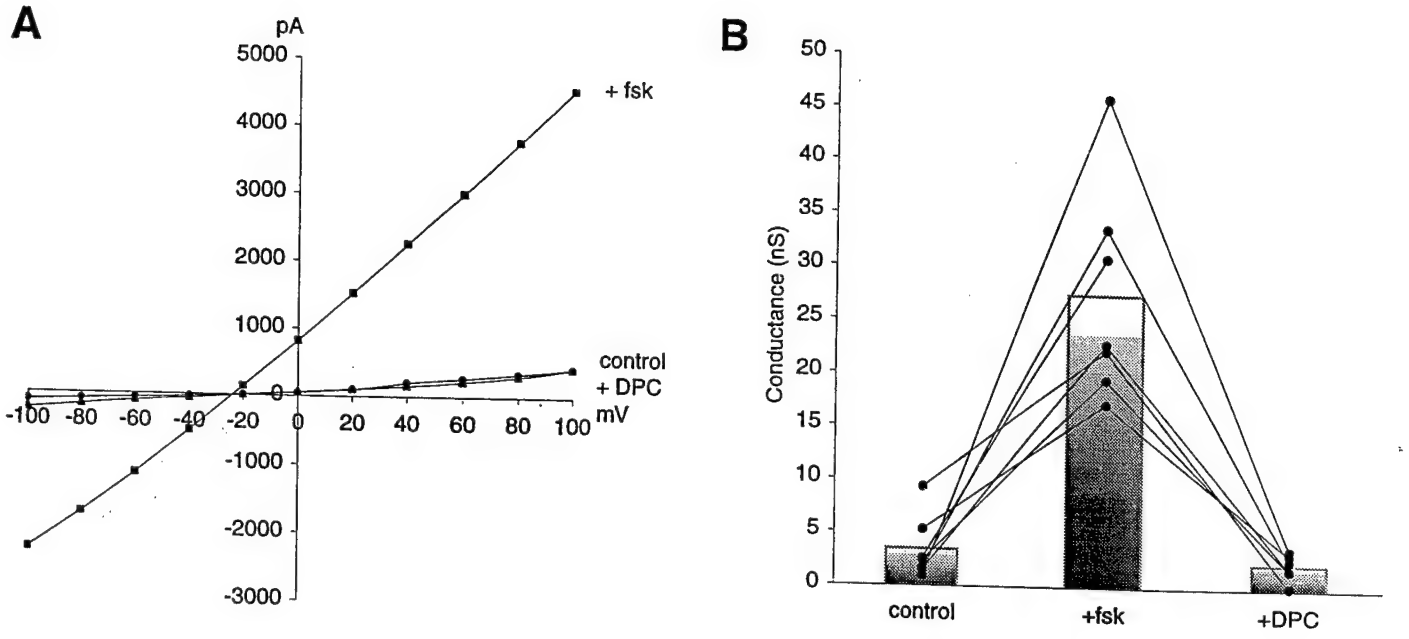

Fig. 7. Effects of forskolin and DPC on whole cell patch clamp current, I-V relationship and conductance. **A**. Whole I-V recording of a resting cell with a basal Cl conductance of 1.7 nS and reversal potential of -20 mV. Upon addition of forskolin, conductance increased to 34 nS, and the reversal potential shifted to -25 mV. Addition of 2 mM DPC decreased the conductance to 2.7 nS and shifted reversal potential back to - 20 mV. **B**. Summary of 7 similar experiments. Average whole cell conductance of resting cells was 3.4 +/- 1.2 nS, stimulated conductance was 27.4 +/- 4.1 nS and after DPC conductance was 2.7 +/- 0.6 nS.

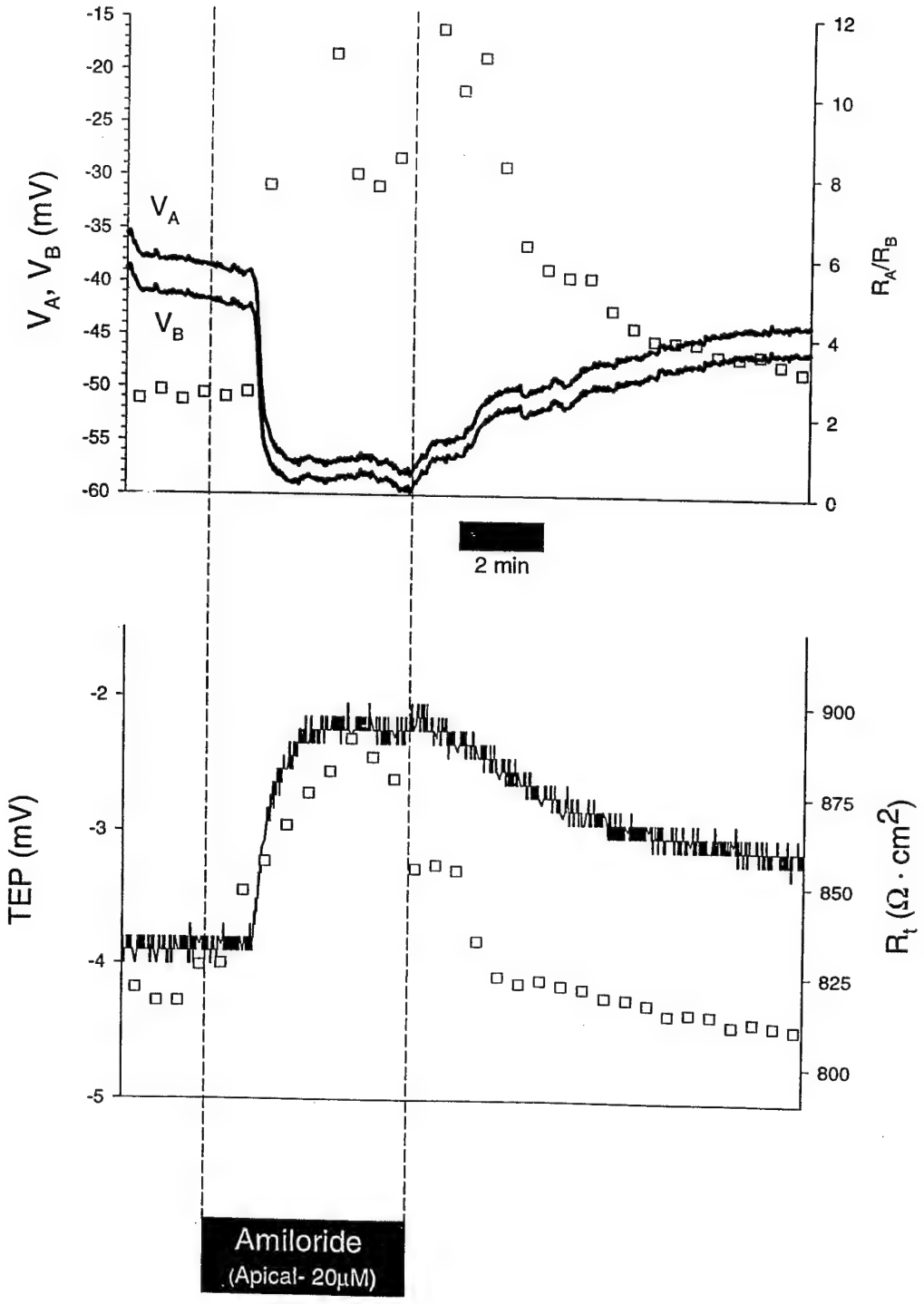

Fig. 8. Effect of amiloride on on cellular and transepithelial electrophysiology. V_A and V_B were both more negative than -45 mV in the control state, and amiloride caused both membrane potentials to hyperpolarize by > 20 mV; R_A/R_B increased from about 2 to > 8; TEP decreased and R_T increased. Effects of amiloride were nearly completely reversible.

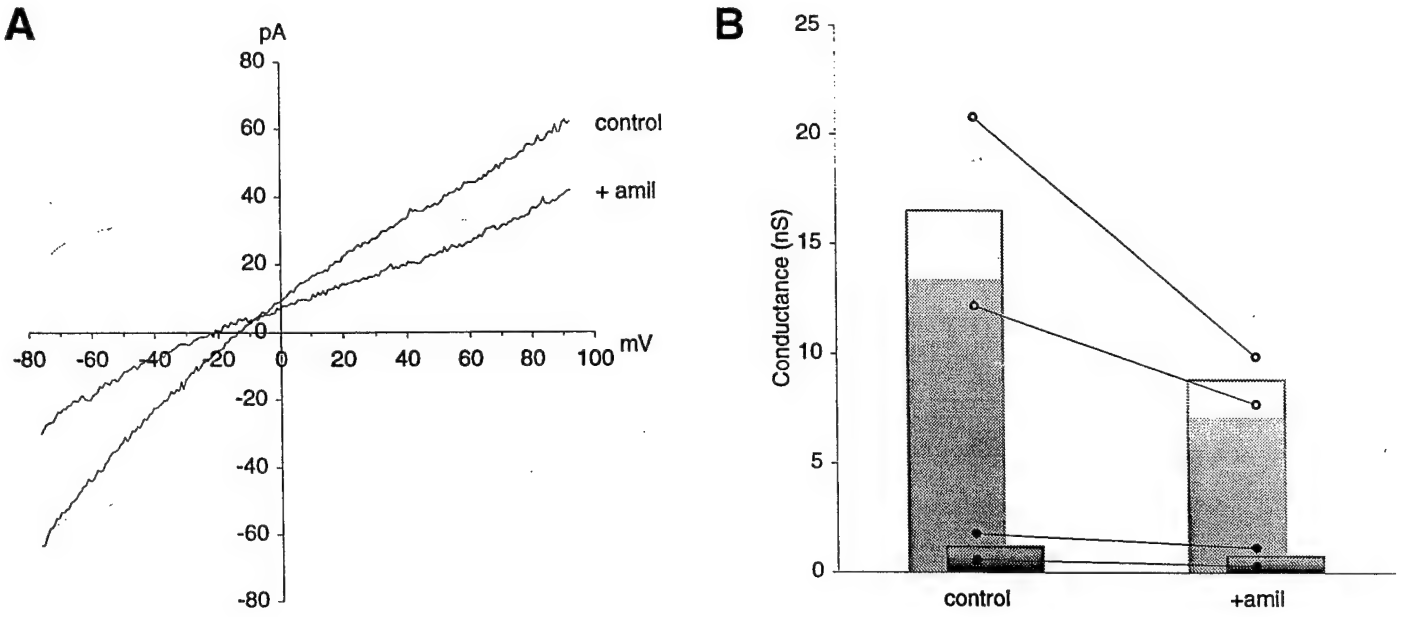

Fig. 9. Effect amiloride on whole cell patch clamp current, I-V relationship and conductance in NaCl and Nagluconate Ringer's. **A.** Whole cell patch recording of a resting cell with a basal conductance in NaCl solution of 0.6 nS and reversal potential of -15 mV. 10 μM amiloride caused conductance to decrease to 0.3 nS and shifted the reversal potential to -22 mV. **B.** Summary of 2 experiments performed with NaCl bathing solutions (open circles) and 2 experiments performed with Nagluconate solutions (filled circles). Average resting conductance in NaCl solution was 16.5 nS and 8.8 nS after amiloride. Average resting conductance in Nagluconate solution was1.2 nS and 0.7 nS after amiloride.

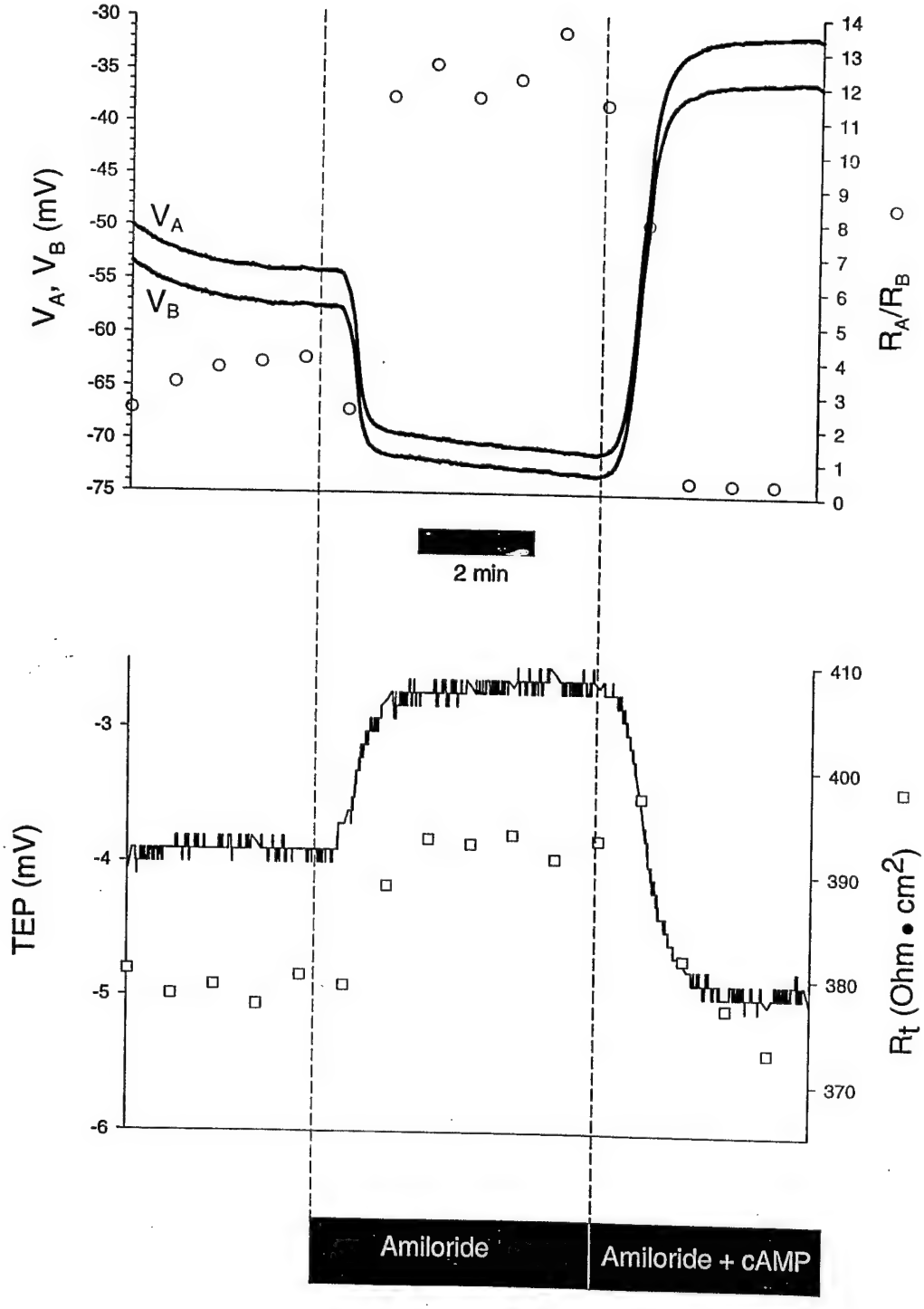

Fig. 10. Effects of cAMP cocktail in the presence of amiloride on cellular and transepithelial electrophysiology. Amiloride caused the characteristic changes of V_A, V_B, R_A/R_B, TEP and R_T, and subsequent addition of cAMP cocktail caused changes of cellular and transepithelial electrophysiology that were very similar to those observed in control cells .

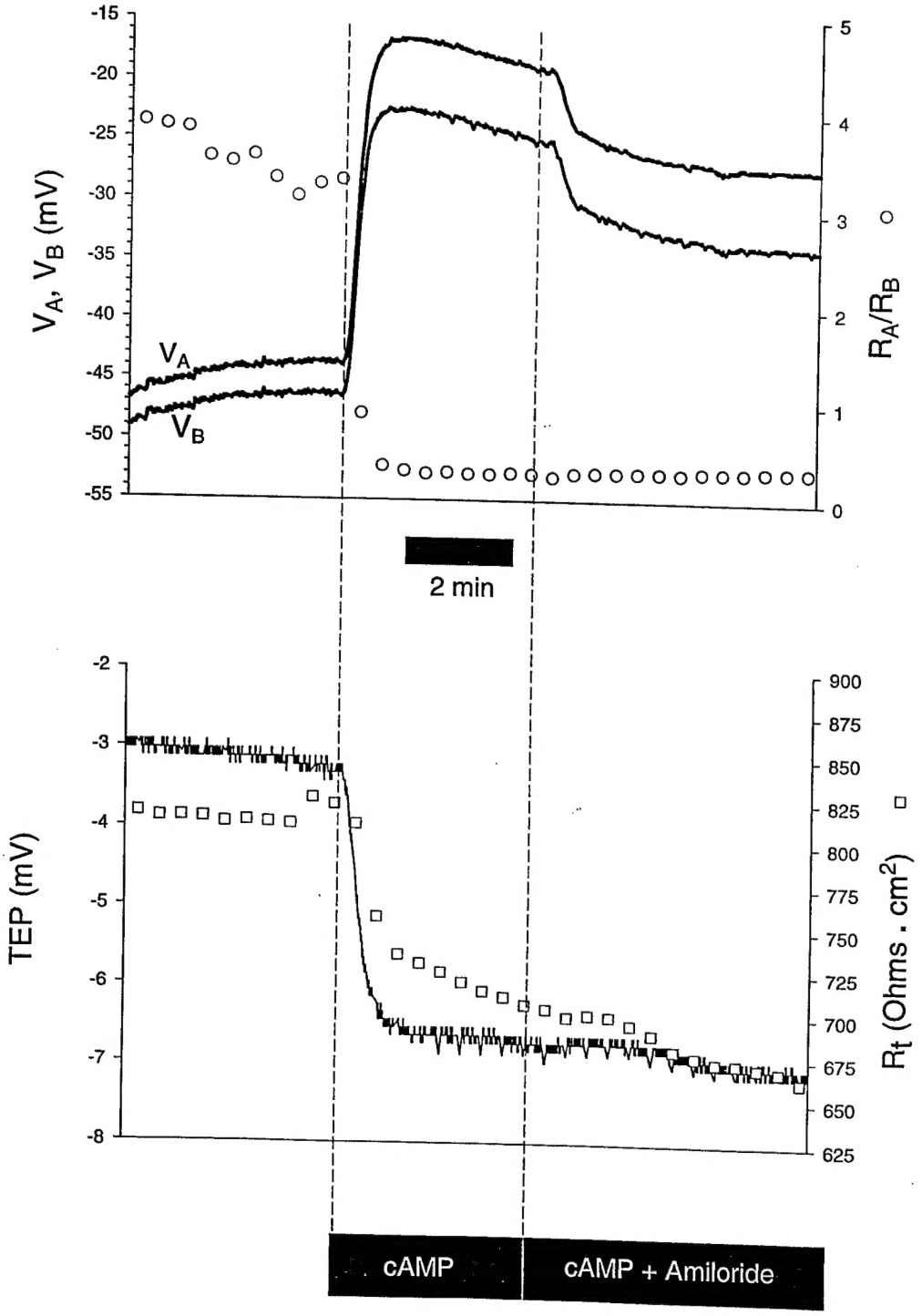

Fig. 11. Effects of amiloride in the presence of cAMP cocktail on electrophysiology. 31EG4 cells were first treated with cAMP cocktail, which caused characteristic depolarization of both V_A and V_B, decrease of R_A/R_B and increase in TEP. Subsequent addition of amiloride had only small effects on all of these parameters.

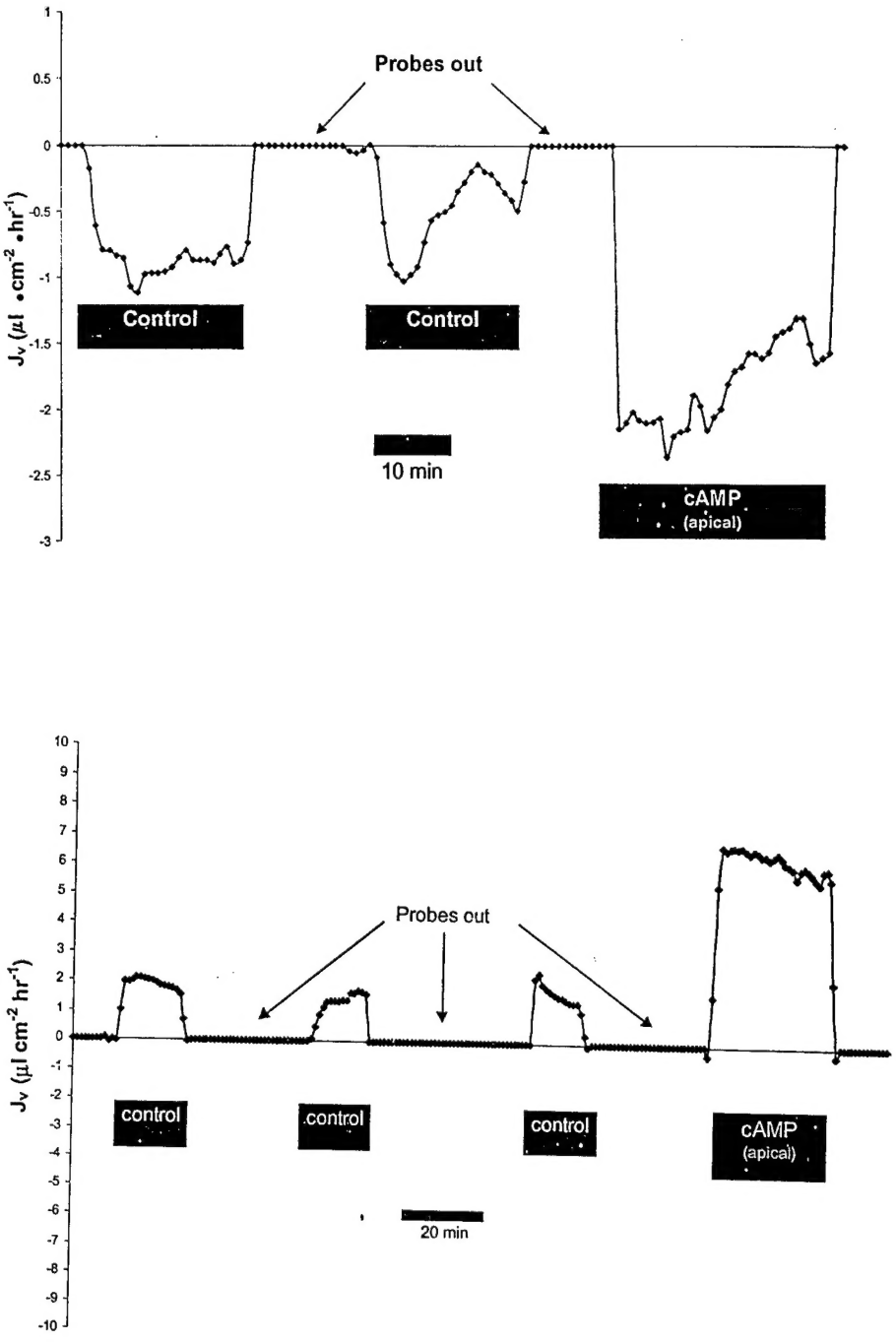

Fig. 12. Effects of cAMP cocktail on fluid transport across 31EG4 monolayers. Secretion is denoted as negative, absorption as positive. **A**. Control monolayer secreted fluid at a rate of 0.5 - 1 µL/cm^2.hr, and this was increased to a steady level of about 5 µL/cm^2.hr by cAMP stimulation. "Probes out" refer to times during the experiment in which the capacitance probes were removed from the chamber to allow for new additions of solutions. **B**. Monolayer absorbed fluid at a rate of 1 - 2 µL/cm^2.hr in the control condition, and this absorption increased to about 5 µL/cm^2.hr during cAMP stimulation.

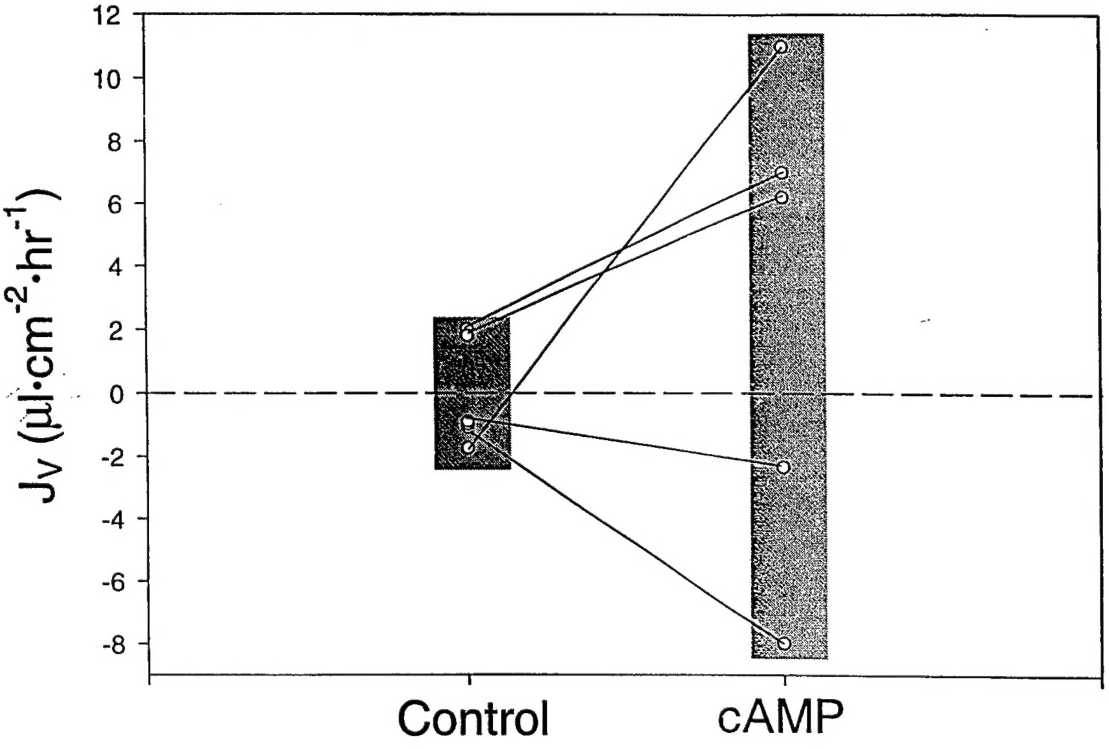

Fig. 13. Summary of effects of cAMP cocktail on fluid transport by 31EG4 cells. Each set of points (connecting control and cAMP-treated conditions) represents one experiment. Control cells either absorbed or secreted fluid at rates of 1-2 2 µL/cm^2.hr.

Final Report:

Manuscript in preparation;

Sasha Blaug , Kevin Hybiske , Terry E. Machen and Sheldon S. Miller., **MAMMARY EPITHELIAL CELLS EXPRESS APICAL ENaC AND CFTR AND TRANSPORT FLUID.**

Meeting Abstract;

North American Cystic Fibrosis Conference, in Seattle, Washington, October 8, 1999.
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